CRYSTALLINE ANHYDROUS Ca-PHOSPHATIDYLSERINE BILAYERS

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SUMMARY

The nature of the Ca-phosphatidylserine complex has been investigated by nuclear magnetic resonance and X-ray diffraction. Ca^{+} binding to the lipid polar group involves the phosphate group and liberates water of hydration from the interbilayer space and from the binding sites of the lipid polar group. Consequently the packing of the lipid polar group becomes tighter and the segmental motion of the phosphate group is reduced. A tightly self-associated, dehydrated ("hydrophobic") Ca-phosphatidylserine complex is formed with crystalline hydrocarbon chains. The overall bilayer structure is retained. The interaction of phosphatidylserine bilayers with Ca^{2+} is equivalent to an isothermal transition of the bilayer from the liquid crystalline to the crystal state.

The interaction of ions with biological membranes is of great importance in the control of the structure and function of such membranes (1,2). Phospholipid bilayers are known to form integral parts of biological membranes and their interactions with ions are at least partly responsible for the ion binding capacity of membranes. Here we study by ^{2}H-NMR and X-ray diffraction the interaction of Ca^{2+} with phosphatidylserine which is one of the negatively charged phospholipids common to many biological membranes (3). We show that the interaction leads to an anhydrous, crystalline, tightly bound complex. This may have important implications in many biological processes involving Ca^{2+}, e.g. nerve excitation, ion translocation, enzymatic activities and the formation of lipoprotein complexes (4).

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Abbreviation: PS = bovine spinal cord 3-sn-phosphatidyl-L-serine

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EXPERIMENTAL

PS (Na^+ salt) and egg phosphatidylcholine were obtained from Lipid Products (South Nutfield, Surrey, U.K.); the lipids were pure by TLC-standards. ζ-potential measurements of the aqueous lipid dispersions showed that the phospholipids were free of polyvalent cations. Lipids and salts (AR grade) were vacuum dried before use. Unsonicated, and sonicated, homogeneous dispersions were prepared as described in ref. (5) and (6), respectively. When required salts were weighed into these dispersions and the sample was rehomogenized (5).

All wideline NMR measurements were performed at 23°C on a Varian Wideline NMR spectrometer operating at 8.13 MHz. Linewidths and quadrupole splittings were measured as described elsewhere (5). ^{31}P-NMR spectra were run at 24.3 MHz on a Perkin-Elmer R22 spectrometer operating in the continuous wave mode. X-ray techniques have been described previously (5).

RESULTS

^{2}H-NMR

Fig. 1a is a ^{2}H-NMR spectrum typical for ^2H_2O-PS mixtures containing only bound water, i.e. for a one phase system with the number n of water molecules/lipid being $\leqslant 140$. At $n \geqslant 140$ two-phase systems are formed consisting of fully hydrated lipid bilayers and excess water (7). The ^{2}H-NMR spectra of such systems consist of a singlet (Fig. 1b) the intensity of which grows with increasing water content. In contrast to PS the two-phase system of fully hydrated egg lecithin in excess water gives a singlet superimposed on a doublet (Fig. 1c, ref. 5) indicating that the free water exchanges only slowly ($<10^2 s^{-1}$) with the water of the lecithin hydration shells. When excess Ca^{2+} is added to hydrated PS bilayers only a sharp singlet is observed in the ^{2}H-NMR spectra of ^2H_2O (Fig. 1d). The linewidth of that singlet $W<40$ Hz is limited by the modulation conditions of the spectrometer. PS precipitated from a sonicated dispersion (5%) in ^2H_2O with $CaCl_2$ ($[Ca^{++}] \geqslant 10^{-3}$M), washed, vacuum dried, remixed with ^2H_2O ($n \sim 20$) and rehomogenized gives a ^{2}H-NMR spectrum very similar

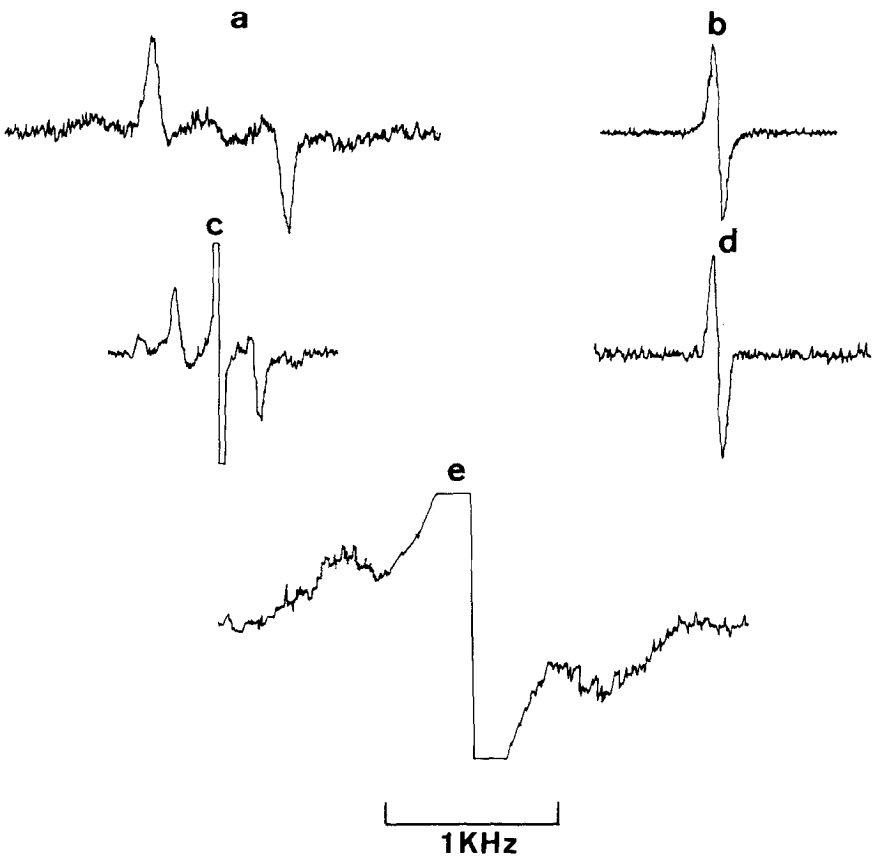

Fig. 1. ^{2}H-NMR spectra of
(a) PS (Na^{+} salt) + $^{2}H_2O$, n = 33;
(b) PS (Na^{+} salt) + $^{2}H_2O$, n = 150;
(c) phosphatidylcholine + $^{2}H_2O$, n = 37;
(d) PS (Na^{+} salt) + $CaCl_2$ + $^{2}H_2O$, n = 20;
(e) PS (Na^{+} salt) + NaCl + $^{2}H_2O$, n = 20;
n = moles $^{2}H_2O$ / mole lipid. Spectra were recorded at 8.13 MHz and 23° C.

to Fig. 1d whereas PS precipitated with NaCl ($[Na^{+}] \gg 1M^{*}$) gives a spectrum consisting of a doublet and a singlet (Fig. 1e).

* The precipitate obtained with 1M NaCl was separated by centrifugation and vacuum dried without washing in order to prevent re-dispersion of the lipid. The dried precipitate thus obtained contained an excess of NaCl.

Table 1

X-ray diffraction of phosphatidylserine

compound	form	long spacing d_{100} (Å)	short spacing (Å)
PS (Na^+ salt)	anhydrous	61.5	4.29
"	aqueous dispersion n = 8	53	—
"	aqueous dispersion n ~ 140	120	—
1,2 distearoyl-3-sn-phosphatidyl-L-serine (from ref. 21)	crystalline α_2-phase	63	4.2
Ca-PS	precipitate	61-62	4.29
Na-PS	precipitate	~60	—

X-ray diffraction

X-ray diffraction analysis shows that PS forms lamellar phases over the whole concentration range (7,8). X-ray powder patterns of anhydrous Na-PS consist of a sharp low angle diffraction line d_{100} = 61.5 Å and higher orders of it characteristic of a lamellar structure. Furthermore, a relatively sharp diffraction at wide angles corresponding to d = 4.29 Å is observed characteristic of crystalline hydrocarbon chains (Table 1). The addition of small amounts of water (n<8) gives lamellar phases with variable long spacings which are usually smaller than 60 Å, e.g. at n = 8 mole ^2H_2O/mole lipid d_{100} = 53 Å. At H_2O contents n>8 the lamellar long spacing d_{100} increases continuously to $d_{100} \geq 120$ Å at n ~140 (~75% ^2H_2O) indicating that a single lamellar liquid crystalline phase is present up to that water con-

tent (cf. ^{2}H-NMR results). The two-phase system at water contents $n \geqslant 140$ gives a low angle diffraction pattern in which the sharp line is replaced by a broad diffuse scattering in the region $0.05 < h < 0.15$ $\mathring{A}^{-1}$ (where $h = 4 \sin\theta/\lambda$, 2θ = scattering angle, λ = X-ray wave length) (7). The scattering curve is remarkably similar to that obtained from sonicated PS dispersions suggesting a close structural similarity between these two systems.

DISCUSSION

The magnitude of the doublet splitting in ^{2}H-NMR spectra of ^2H_2O is a measure of the degree of anisotropic motion of the ^{2}H-O bond (5,9,10). The doublet in Fig. 1a arises from ^2H_2O molecules being present in different hydration shells of PS and exchanging fast ($>10^4 s^{-1}$) between these different environments (5). The residual singlet representing isotropically tumbling water is probably due to ^2H_2O hydrating lipids present in defects of the regular multilamellar packing. The singlet observed with the two-phase system (Fig. 1b) is interpreted to mean that the excess free water at $n \geqslant 140$ giving rise to singlet spectra is in fast exchange with the water of the lipid hydration shells. One possible explanation is that in excess water the multilamellar structures no longer exist (cf. ref. 7,11 and X-ray results) and that the structure formed allows for fast exchange to take place between free and bound water. The singlet observed in the presence of excess Ca^{2+} (Fig. 1d) is regarded as an indication that Ca^{2+} interacting with PS displaces both Na^+ (12,24) and water of hydration forming a dehydrated Ca-complex. The water liberated upon the addition of Ca^{2+} is either free or present in the hydration shell of excess Ca^{2+} and as such tumbles isotropically giving rise to a singlet. The Ca-PS complex is thus mostly dehydrated, if not anhydrous. Taking into account that the linewidth of the ^{2}H doublet increases as $n \rightarrow 0$ there is a limit in the detection of bound water. It is estimated that n = 3 is the lower limit for detection of hydration and hence the hydration of the Ca-PS complex cannot exceed 3 mole H_2O/mole lipid. However, by analogy with the reaction of Mn^{2+} and PS (13), it is very likely that the Ca^{2+}-PS complex is anhydrous. ^{31}P-NMR measure-

ments show that the linewidth of the ^{31}P signal of unsonicated PS dispersions is completely broadened out into the baseline when the Ca complex is formed. This indicates that the interaction of Ca^{2+} leads primarily to a tighter packing and immobilisation of the polar group (14-20); the effect is propagated to the hydrophobic part of the bilayer leading to the crystallisation of the hydrocarbon chains.

In contrast to Ca^{2+}, excess Na^+ does not form an anhydrous precipitate as evident from the doublet splitting observed (Fig. 1e). Furthermore, different from the Ca-PS complex the precipitate produced by excess Na^+ is readily redispersed in excess ^2H_2O.

The NMR work is consistent with the X-ray diffraction data. The Ca-PS complex precipitated from sonicated lipid dispersions has a multilamellar structure characterized by a long spacing d_{100} = 61-62 Å and a relatively sharp diffraction line at wide angles corresponding to d = 4.29 Å (Table 1) characteristic of crystalline hydrocarbon chains. The X-ray diffraction pattern of Ca-PS closely resembles that reported for the crystalline α_2-phase of synthetic 1,2-distearoyl-3-sn-phosphatidyl-L-serine (21) which is characterized by a long spacing of 63 Å and a short spacing of 4.2 Å (22). Hence the addition of Ca^{2+} to liquid crystalline, lamellar phases of PS has the same effect as cooling the sodium salt of PS below the transition temperature. Ca^{2+} thus induces a reversible, isothermal phase change (Fig. 2).

$$\text{liquid crystal} \quad \underset{+\ Na^+}{\overset{+\ Ca^{2+}}{\rightleftharpoons}} \quad \text{crystal}$$

the direction of the reaction being controlled by the Ca^{2+} activity of the medium.

The behaviour of the Ca-PS complex is contrasted by that of PS precipitated by excess $[Na^+] \geqslant 1M$. The X-ray diffraction pattern shows a sharp diffraction typical for multilamellar structures with long spacings of ~ 60 Å and usually some weaker diffraction lines from multilamellar structures of different dimensions (cf. ref. 7). The observation of different long spa-

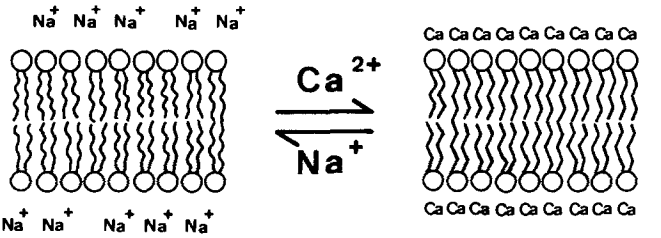

Fig. 2. Schematic diagram showing the effect of Ca^{2+} on the packing of the hydrocarbon chains of phosphatidylserine bilayers. Ca^{2+} displaces Na^{+} present in the electrical double layer forming a tight complex with the lipid polar groups. The tight packing in the polar group is then propagated to the hydrocarbon region leading to the crystallisation of the hydrocarbon chains. The stoichiometry of the Ca-phosphatidylserine complex is arbitrarily represented as 1 (cf. 24).

cings is explained in terms of the coexistence of bilayers differing in the extent of hydration, indicating that under these conditions the bilayers do at least partially retain water of hydration. The precipitation of phosphatidylserine at $[NaCl] > 1M$ is a typical "salting out" effect involving charge neutralisation and consequently a reduction in the repulsive double layer potential between adjacent bilayers accompanied by a partial dehydration of the lipid polar groups.

The addition of Ca^{2+} thus triggers the following sequence of events: as a result of the binding of Ca^{2+} to the lipid polar group the packing of that part of the molecule becomes tighter and the segmental motion of the phosphate group is reduced; the concomitant reduction in the repulsive bilayer potential produces a reduction in the bilayer spacing: water is extruded from the interbilayer space. Ca^{2+} eventually replaces water of hydration from the phosphate group (cf. ref. 23) forming a tight, anhydrous Ca-lipid chelate with crystalline hydrocarbon chains (Fig. 2).

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